

2 **Discovery of a novel ejaculation mechanism in danceflies (Diptera: Empididae), with**  
3 **implications for genital elongation**

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8

9 Abstract

10 Genitalia are known to evolve rapidly and are among the most variable structures in insect  
11 morphology, making them a target of active research. However, function and evolutionary  
12 significance of internal genital structures remain less well understood. Here, we report the  
13 morphology and mechanism of a novel ejaculatory system that has evolved in the dancefly genus  
14 *Rhamphomyia* (Insecta: Diptera: Empididae). Using synchrotron  $\mu$ CT technology, we examined  
15 male genitalia of five dancefly species and identified an ejaculatory system resembling a leverage  
16 hydraulic jack, which is thought to have derived from a plunger-like pumping system. This jacking  
17 system amplifies the applied muscle power by up to 4.2 times, allowing the system to produce the  
18 same pumping power with much smaller muscles. However, the volume of the pumping muscle in  
19 the jacking system is comparable to that of the plunger system, indicating a significant increase in  
20 ejaculation power in this genus. We hypothesize that the greater pumping power evolved through  
21 sexual selection favoring strong ejaculation to rapidly pass semen through a thin and elongated  
22 phallus and spermathecal duct.

23

24 Keywords sperm pump • evolutionary novelty • lever system • genitalia • sexual selection

## 25 **Introduction**

26 The lever system is highly effective, especially when the effort arm (EA) is longer than the load  
27 arm (LA), as it amplifies the applied force according to the ratio of EA to LA. This power  
28 efficiency has led to the widespread use of lever systems in human tools, such as scissors, can  
29 openers, and hydraulic jacks. Lever systems are also commonly observed in insects, as in  
30 mechanisms like wing flapping hinges, jaw chewing apparatuses, and leg articulations (Chapman  
31 1998). These systems are thought to have evolved simultaneously with the origin of these structures.  
32 There are instances of existing lever systems being strengthened and achieved new function, as seen  
33 in the wasp's abdomen and the beetle's horn (Hashimoto 1996; Weber et al. 2023). However, if not  
34 entirely absent, it is a very rare phenomenon that a pre-existing mechanism is completely replaced  
35 by a lever mechanism in insects while retaining the original function.

36 Male flies (Diptera) possess a pumping structure at the base of the phallus, known as a  
37 sperm pump, which acts as an ejaculator of semen. The sperm pump comprises a cylindrical sperm  
38 sac and an apodeme, or the ejaculatory apodeme. The ejaculatory apodeme, an endoskeleton  
39 projecting anteriorly from the sperm sac, provides an attachment site for muscles that generate the  
40 pumping power necessary for ejaculation. In its ancestral form, the ejaculatory apodeme moves like  
41 a piston that directly compresses the sperm sac like plunger (Figs. 1A, 2A) (Downes 1965;  
42 Ovchinnikova 1989). Two or three pairs of muscles attached to the ejaculatory apodeme are aligned  
43 nearly parallel to its pushing axis, acting as direct compressors of the ejaculatory apodeme. Because  
44 there is no muscle functioning as a decompressor, the compressed sac presumably returns to its  
45 original condition by its own resilience (Downes 1965; Ovchinnikova 1989).

46 Here, we report the morphology and function of a novel lever-jacking system identified in  
47 the sperm pump of a genus of danceflies (Empididae), a family representing a basal lineage of the  
48 higher Diptera (suborder Brachycera). Using  $\mu$ CT analyses, we confirmed that, except for species  
49 of a particular group, two pairs of muscles attached to the ejaculatory apodeme are aligned nearly  
50 parallel to its pushing axis (Trehen 1961, 1962). These muscles likely act as direct compressors as  
51 has been reported in both lower and higher Diptera (Downes 1965; Ovchinnikova 1989). This  
52 suggests that these empidid species retain a pumping system similar to the dipteran ground plan  
53 condition and have no decompressor muscles.

54 In contrast, the novel pumping system we identified involves a lever mechanism, where  
55 one muscle pair functions as a compressor and the other as a decompressor. Further modifications  
56 similar to a hydraulic jack were also detected. We examined the sperm pump structures and  
57 associated muscles of five empidid species to elucidate the evolutionary novelties related to the

58 origin of this lever-jacking system. Additionally, we discuss the evolutionary background of the  
59 novel ejaculatory system in relation to genital elongation in danceflies.

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## 62 **Material and methods**

63 The following five dancefly species (Insecta: Diptera: Empididae) were subjected for the present  
64 examinations. Tribe Empidini: *Rhamphomyia* (*Calorhamphomyia*) *longistigma*, *Rhamphomyia*  
65 (*Rhamphomyia*) sp. (*sulcata* group), *Empis* (*Euempis*) sp. (*tessellata* group), and *E.* (*Polyblepharis*)  
66 *multinodosa*; tribe Hilarini: *Hilara* sp. The subgeneric name, with an abbreviated generic name, is  
67 used to refer to each species in the following lines. Samples were fixed with hot water then  
68 preserved in 80% ethanol. Samples were dehydrated with 80–100% ethanol in ascending order  
69 before critical point drying (EM CPD300, Leica, Wetzlar, Germany). Samples (one specimen for  
70 each species) were scanned using synchrotron  $\mu$ CT at the BL20XU or BL20B beamlines (Uesugi  
71 et al. 2012) of the Super Photon ring-8 GeV (SPring-8: Hyogo, Japan). We used ITK-SNAP 3.6  
72 (Yushkevich et al. 2006) to obtain 3D representations. Muscle volume was calculated based on the  
73 pixel numbers in the reconstructed 3D models. The length of the hind femur was used as an index  
74 of body size. The pushing axis was defined as a line connecting the anterior meeting point of the  
75 M1/M2 muscles and the middle of the base of the apodeme (for the direct compressing system) or  
76 the tip of the load arm (for the lever systems). The power direction produced by each muscle was  
77 estimated by connecting the midpoint of its origination and insertion sites. A detailed account of the  
78 methods is given in the electronic supplementary material. Raw data and reconstructed 3D model of  
79 all species examined are available from FigShare at DOI: 10.6084/m9.figshare.26377348.

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## 82 **Results and discussion**

83 The empidid sperm pump is composed of the following elements in all five species (Fig. 1): the  
84 enlarged sperm sac (ss) connected dorsally to the testis via a tube (tu) and ventrally to the inner tube  
85 of aedeagus (ad); the ejaculatory apodeme (ea) extending from the anterior surface of the sperm sac  
86 and consisting of a vertical and a horizontal plate, forming a cross-section a "+" shape; and two sets  
87 of muscles (dorsal M1 and ventral M2) originating from the ejaculatory apodeme (both vertical and  
88 horizontal plates) and attaching to the 9th sternum extending and continuing to the sperm sac. These  
89 structural and muscular characters (Fig. 1) are consistent with previous observations of the other

90 empidid species (Trehen 1961, 1962; Ulrich 1972).

91 In all species except for those in the genus *Rhamphomyia* (subgenera *Rhamphomyia* and  
92 *Calorhamphomyia*), the ejaculatory apodeme extends anteriorly, with its base tightly united with the  
93 sperm sac (Fig. 1A). Both ejaculatory muscles are large and comparable in volume (ca. 1:1 to 2:1 in  
94 M1:M2 volume ratio: Table 1) and are arranged nearly parallel to the pushing axis of the  
95 ejaculatory apodeme (Fig. 1A). The similarity in volume and nearly symmetrical arrangement of  
96 the two ejaculatory muscles suggests they work together to provide direct compressing power to the  
97 sperm sac, functioning like a plunger (a direct compressing system, or DC system: Fig. 2A). No  
98 decompressor muscle was detected in the DC system (Fig. 1A), suggesting that the sperm sac  
99 returns to its original state by the resilience of the sac sclerite (Downes 1965; Ovchinnikova 1989).  
100 This empidid ejaculation system agrees with the ancestral plunger-like mechanism of the dipteran  
101 sperm pump (Downes 1965; Ovchinnikova 1989). Lever-like modifications of the ejaculatory  
102 apodeme have been previously mentioned as an autapomorphy of the superfamily Empidoidea,  
103 without examining muscle conditions (Cumming et al. 1995; Sinclair 2000; Sinclair and Cumming  
104 2006). However, our present examinations revealed that at least some dancefly species  
105 (representing two tribes, Empidini and Hilarini) retain the dipteran ancestral pumping system.

106 In contrast, the M2 muscle in *R. (Rhamphomyia)* is significantly smaller than M1  
107 (approximately 1/9 in volume; Table 1) and is arranged nearly perpendicular to the M1 muscle (Fig.  
108 1B). The M1 muscle is nearly parallel to the pushing axis of the apodeme, but the M2 muscle is  
109 arranged at a right angle to this axis (Fig. 1B), indicating that it cannot function as a direct  
110 compressor. In the examined specimen, the sperm sac was almost completely compressed, but the  
111 base of the aedeagal tube (the articulation point) remained nearly undeformed (Fig. 1B). The  
112 reconstructed 3D model showed that the apodeme is united to the sperm sac, and there is an  
113 articulation between the posteroventral corner of the apodeme and the base of the inner tube of  
114 aedeagus (asterisk in Fig. 1B). These muscular and structural features suggest that the apodeme  
115 functions as a lever (the lever-compressing system: LC), with the articulation serving as a fulcrum,  
116 and that M1 acts as a compressor while M2 functions as a decompressor (Fig. 2B). The ejaculatory  
117 apodeme (effort arm: EA) and its base (load arm: LA) are arranged in an L-shape, with the  
118 articulation point at the corner (a type 1 lever: Figs. 1B, 2B). The formation of the LC system  
119 required not only the structural innovations mentioned above but also changes in the muscle control  
120 system (e.g., impulse pattern), transitioning from synchronous M1-M2 compressions to alternating  
121 compressions (Fig. 2).

122 The EA to LA length ratio in *R. (Rhamphomyia)* is approximately 1:1.25 (Fig. 1B). A lever  
123 system can amplify pumping power according to EA/LA. However, in *R. (Rhamphomyia)*, EA/LA

124  $< 1$ , so this system does not amplify muscle power. Nevertheless, the LC system in *R.*  
125 (*Rhamphomyia*) is probably more efficient mechanically than the DC system of other danceflies or  
126 dipterans. The absence of a decompressor muscle in the DC system implies that the sperm sac must  
127 have strong resilience to recover from compression. In contrast, the LC system includes a  
128 decompressor muscle, eliminating the need for strong resilience in the sperm sac. Compressing a  
129 highly resilient sperm sac requires much greater muscle power than compressing a sac with low  
130 resilience, likely making the LC system in *R. (Rhamphomyia)* more efficient mechanically  
131 compared to the DC system in other dipterans (Downes 1965; Ovchinnikova 1989).

132 Further modification of the ejaculatory system, likely evolved via the LC system, was  
133 detected in *R. (Calorhamphomyia)* (Fig. 1C). The ejaculatory apodeme extends anteroventrally (Fig.  
134 1C) rather than anteriorly (Fig. 1AB), with its base separated from the sperm sac by a membranous  
135 region (Supplementary Movie), except for the ventral tip firmly articulated with the base of the  
136 aedeagal tube (asterisk in Fig. 1C). The apodeme also lacks the lower half of the vertical plate  
137 (attachment site of the M2 muscle), while the horizontal plates extend ventrolaterally, forming a  
138 cross-section resembling an inverted "Y" shape. Two sets of muscles were confirmed, but the M2  
139 muscle is extremely reduced in volume (approximately 1/370 of M1: Fig. 1C, Table 1) and attaches  
140 to the lower crotch of the inverted Y-shaped apodeme (Fig. 1C). As in *R. (Rhamphomyia)*, M2 does  
141 not function as a compressor but is assumed to act as a decompressor (Fig. 1C, Supplementary  
142 Movie). Compression by M1 causes a dorsal shift of the apodeme, but due to its separation by a  
143 membrane, it does not modify the sperm sac itself (Supplementary Movie). Instead, the base of the  
144 apodeme surrounded by a membrane pushes the semen, like a hydraulic jack (a lever-jacking  
145 system, or LJ system). In this species, the effort arm is much longer than the load arm, and the  
146 pumping power produced by this LJ system can be estimated as approximately 4.2 times the  
147 applied muscle force (Fig. 1C). Additionally, the membrane surrounding the base of the apodeme  
148 allows the jacking system to operate with lesser power than the compressing systems. Our  
149 experiments also showed that the apodeme of *R. (Calorhamphomyia)* can be moved very easily and  
150 has little resiliency (Supplementary Movie). This suggests that the LJ systems can generate more  
151 effective pumping power with much smaller muscles compared to the DC or LC systems.

152 However, despite the power efficiency of LC and LJ systems compared to the DC system,  
153 the total muscle volume of the sperm pump system in *R. (Rhamphomyia)* (LC system) and *R.*  
154 (*Calorhamphomyia*) (LJ system) is approximately comparable to (or even larger than) that of other  
155 danceflies with the DC system (Fig. 3). This implies that *R. (Rhamphomyia)* and *R.*  
156 (*Calorhamphomyia*) ejaculate semen with much greater force than other empidids. We assumed two  
157 possibilities which likely interact to drive the evolution of leverage pumping systems: (1) The

158 aedeagus of *R. (Rhamphomyia)* and *R. (Calorhamphomyia)* is thinner, longer, and more sinuous  
159 compared to that of other danceflies examined (Fig. 2CD). Semen is a viscous liquid that requires  
160 significant force to be pumped through a thin and long tube, necessitating greater muscle power  
161 (Matsumura et al. 2019). Elaborate genital structures are generally thought to be formed as a result  
162 of sexual selection (Eberhard 1985). In danceflies and other dipterans, the length of the female's  
163 spermathecal duct typically corresponds to that of the male aedeagus (Downes 1965; Akbar et al.  
164 2022), suggesting a coevolutionary relationship between male and female genitalia. This further  
165 implies that the elongated and sinuous aedeagus of *R. (Rhamphomyia)* and *R. (Calorhamphomyia)*  
166 likely evolved due to sexual selection. If seminal viscosity acts as a physical constraint on genital  
167 evolution, the formation of lever-type ejaculatory mechanisms may have enabled males to respond  
168 to sexual selection favoring elongated and sinuate aedeagus; (2) Strong pumping power itself might  
169 be favored and evolved through sexual selection. Many dancefly females only allow males to  
170 copulate while they are eating a nuptial gift (Svensson et al. 1990). In the hangfly *Hylobittacus*  
171 *apicalis* (Mecoptera), females also accept mating only during the consumption of a nuptial gift. It  
172 has been reported that the narrow and elongated spermathecal duct in this hangfly hinders rapid  
173 sperm transfer from males, and only males offering a large prey item can transfer enough sperm to  
174 ensure their paternity (Thornhill 1976). If this is also the case for danceflies, strong sperm pumping  
175 may be also advantageous for males to transfer sperm quickly and certainly.

176         The lever-like ejaculatory apodeme was once considered an autapomorphy of Empidoidea  
177 (Cumming et al. 1995; Sinclair 2000; Sinclair and Cumming 2006). Therefore, although the lever  
178 system was only detected in the genus *Rhamphomyia* in this study, it might be more widely  
179 distributed in the superfamily. For example, genital photographs of *R. (Pararhamphomyia)* showed  
180 a pumping system similar to LJ system (Barták et al. 2015), which has been confirmed here only in  
181 *R. (Calorhamphomyia)*. Observing the sperm pump system in a wide variety of danceflies is  
182 necessary to uncover the diversity, transitional patterns, and evolutionary background of the  
183 leverage pumping system. The mating system of danceflies are also highly diverse and complex,  
184 including swarming, nuptial gifting, active multiple couplings by female, and sex-role reversals  
185 (e.g., Svensson et al. 1990; Funk and Tallamy 2000). These factors are crucial for understanding  
186 genital evolution and sexual selection in danceflies. Establishment of a robust phylogeny for  
187 Empididae is also a key to elucidating morphological evolution and its relation to the behavioral  
188 traits within this family. It also remains unclear why the evolution of a highly efficient lever system  
189 did not lead to a reduction in the volume of pumping muscles, necessitating detailed functional and  
190 behavioral analyses. While the evolution of elaborate genitalia as a consequence of sexual selection  
191 is a very actively studied area, the relationship between the structural evolution and the ejaculating  
192 muscles and/or seminal hydrodynamics has less attracted (Matsumura et al. 2019). It should also be

193 noted that female dipterans also have a sperm uptake system at their spermathecal duct (Cumming  
194 and Wood 2017). The evolution of empidid genital structures could provide intriguing insights into  
195 the physical constraints on genital evolution.

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203

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205

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254

255 **Fig. 1.** The sperm pump of (A) *E. (Euempis)*, (B) *R. (Rhamphomyia)*, (C) *R. (Calorhamphomyia)*.  
256 Left ones show full structure (muscles in half-translated), and right ones omit the muscles and  
257 the foreground sac sclerite. Dotted arrow indicates the direction of the compression of each  
258 muscle or pushing direction of the ejaculatory apodeme. Abbreviations: \* – fulcrum; ad –  
259 aedeagal tube; ea – ejaculatory apodeme; EA/LA – effort/load arm; M1/2 – muscle #1/#2; ss  
260 – sperm sac; tu – tube to the testis. Scale = 0.1 mm.

261 **Fig. 2.** (AB) Schematic illustrations showing the mechanism of (A) the direct compressing (DC)  
262 and (B) leverage compressing/ leverage jacking systems (LC/LJ). Left ones show relaxed  
263 condition and right ones show compressed condition. Note that LC and LJ are mainly differed  
264 in the ejaculatory apodeme connected with (LC) or separated from (LJ) the sac sclerite. See  
265 Fig. 1 for abbreviations. (CD) The male terminalia of (C) *E. (Euempis)* (with simple aedeagus  
266 and DC system) and (D) *R. (Calorhamphomyia)* (with elongated and sinuate aedeagus and LJ  
267 system). Blue arrow heads indicate the tip of aedeagus.

268 **Fig. 3.** Relationships between the hind femur length and the sperm pumping muscle volume of each  
269 species examined.

270

271 **Table 1.** Measurements of the sperm pump muscles and hind femur.

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273 **Supplementary Movie.** Manipulation of the lever-jacking sperm pumping system of *Rhamphomyia*  
274 (*Calorhamphomyia*) *longistigma*.





